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Activation of the histaminergic H_3 receptor induces phosphorylation of the Akt/GSK-3β pathway in cultured cortical neurons and protects against neurotoxic insults

Chiara Mariottini,*[,1] Tania Scartabelli,† Gerold Bongers,‡[,1] Silvia Arrigucci,† Daniele Nosi,§ Rob Leurs,‡ Alberto Chiarugi,† Patrizio Blandina,† Domenico E. Pellegrini-Giampietro† and Maria Beatrice Passani†

*Dipartimento di Scienze Fisiologiche, Universitá di Firenze, Firenze, Italy

†Dipartimento di Farmacologia Preclinica e Clinica, Universitá di Firenze, Firenze, Italy

‡Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

§Dipartimento di Anatomia, Istologia e Medicina Legale, Firenze, Italy

Abstract

Stimulation of histamine H_3 receptors (H_3R) activates $G_{i/o}$-proteins that inhibit adenylyl cyclase and triggers MAPK and phospholipase A_2. In a previous study, we showed that H_3R-mediated phosphorylation of Akt at Ser473 occurs in primary cultures of rat cortical neurons, but neither the downstream targets nor the function of such activation were explored. In this report we address these questions. Western blotting experiments showed that H_3R-mediated activation of Akt in cultured rat cortical neurons was inhibited by LY 294004 and U0126, suggesting that it depends on phosphoinositide-3-kinase and mitogen-activated protein kinase kinase. H_3R activation phosphorylated, hence inactivated, the Akt downstream effector glycogen synthase kinase-3β, increased the expression of the antiapoptotic protein Bcl-2 and protected cultured rat and mouse cortical neurons from neurotoxic insults in a dose-dependent manner. All these effects were inhibited by the H_3R antagonist inverse/agonist thioperamide. Mouse cortical cells expressed H_3R as revealed by immunostaining experiments, and stimulation of H_3R phoshorylated Akt and decreased caspase 3 activity. Hence, we uncovered a yet unexplored action of the H_3R that may help understand the impact of H_3R signaling in the CNS.

Keywords: apoptosis, Bcl-2, excitotoxicity, immepip, neuroprotection.

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The histamine H_3 receptor (H_3R) was originally described as an autoreceptor regulating synthesis and release of histamine (Arrang *et al.* 1983), and subsequently it was found to be present on pre-synaptic sites where it modulates the release of several neurotransmitters (Schlicker *et al.* 1994). Post-synaptic H_3R are also located on the perikarya of many neuronal populations (Pillot *et al.* 2002), but their physiological role is not completely understood. Stimulation of the H_3R activates several intracellular pathways including $G_{i/o}$-dependent inhibition of adenylate cyclase, activation of phospholipase A_2, as well as inhibition of the Na^+/H^+ exchanger, and of K^+-induced Ca^{2+} mobilization (Leurs *et al.* 2005). We recently demonstrated that in transfected SK-N-MC cells, the human H_3R modulates the phosphorylation of Akt and one of its substrates, glycogen synthase kinase 3β (GSK-3β), both in a constitutive and agonist-dependent fashion (Bongers *et al.* 2007a). The H_3R-mediated phosphorylation of Akt at Ser473 occurs also in primary rat cortical neurons and in rat striatal slices (Bongers *et al.* 2007a). In this work although, neither the physiological

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Address correspondence and reprint requests to M. Beatrice Passani, Dipartimento di Farmacologia Preclinica e Clinica, Viale Pieraccini 6, 50139 Firenze, Italy. E-mail: beatrice.passani@unifi.it

[1]The present address of Chiara Mariottini and Gerold Bongers is the Mount Sinai School of Medicine, Department of Pharmacology and Biological Chemistry,1 G. Levy Place, New York, NY 10029, USA.

Abbreviations used: CREB, cAMP response element binding; DIV, days in vitro; ED, embryonic day; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; GSK, glycogen synthase kinase; H_3R, H_3 receptor; LDH, lactate dehydrogenase; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NDS, normal donkey serum; PBS, phosphate-buffered saline; PI3K, phosphoinositide-3-kinase; RAMH, R-α-methylhistamine; TBS, Tris-buffered saline; TX, Triton X-100.

significance of H_3R-induced Akt activation nor the intracellular events associated with Akt phosphorylation were studied. In the CNS, the Akt/GSK-3β axis plays a prominent role in several brain functions. The enzymatic activity of GSK-3β promotes apoptosis in a wide variety of conditions, such as trophic factor withdrawal and neurotoxic insults (reviewed in Li *et al.* 2002). Activation of Akt phosphorylates GSK-3β and inhibits its activity, hence promoting cell survival. Evidence links increased GSK-3β activity with Alzheimer's disease (Jope and Johnson 2004); in a mouse model of this neurodegenerative disease, down-regulation of GSK-3β-conditional over-expression diminishes neuronal death and cognitive deficits (Engel *et al.* 2006). Reduced Akt function has been reported in schizophrenic patients (Emamian *et al.* 2004), and antipsychotic drugs that antagonize the dopaminergic D_2 receptor activate the Akt/GSK-3β axis in the mouse brain (Kang *et al.* 2004; Li *et al.* 2007).

Herein, using western blot analysis we characterize the H_3R-dependent activation of the Akt/GSK-3β axis in primary rat cortical neurons, which differs in several aspects from the properties of the transfected human H_3R. We investigated the ability of the H_3R to modulate the activity of antiapoptotic pathways and to prevent neuronal damage in two distinct models of neurotoxicity. Our results suggest a neuroprotective effect of H_3R stimulation and may have relevance in the treatment of, e.g. ischemia or neurodegenerative diseases such as schizophrenia or Alzheimer's disease.

Experimental procedures

Materials

Neurobasal medium, Eagle's minimal essential medium, and all tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, Mi, Italy). GoTaqFlexi DNA polymerase's reaction kit was obtained from Promega (Madison, WI, USA). Bovine serum albumin fraction V and anti β-actin antibodies were obtained from Sigma (Milano, Italy). Skimmed milk powder was purchased from Bio-rad (Hercules, CA, USA). Immepip and (R)-α-methylhistamine (RAMH) were synthesized at Vrije Universiteit Amsterdam; thioperamide, MK-801 and U0126 were purchased from Tocris Bioscience (Bristol, UK); NMDA was purchased from Sigma; the Akt inhibitor (1L-6-hydroxymethyl-chiroinositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) was obtained from Calbiochem (Merck Biosciences Ltd, Nottingham, UK). Antibodies recognizing phospho-Akt (Ser473), phospho GSK-3β (Ser9), phospho-cAMP response element binding (CREB) (Ser133), Bcl-2, and LY 294002 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies recognizing the H_3R were kindly provided by Drs Chazot and Shenton (Durham University, UK); anti-glial fibrillary acidic protein (GFAP) antibodies were from Sigma. Anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were from Pierce (Rockford, IL, USA). Donkey anti-rabbit Cy3-conjugated secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA); goat anti-rabbit Alexa Fluor 488-conjugated secondary antibodies were from Molecular Probes (Eugene, OR, USA).

Rat cortical neurons in culture

Primary cortical neurons were prepared from embryonic day (ED) 17 Sprague–Dawley rats (Harlan, Italy) as described in Bongers *et al.* (2007a) (details in 'Supporting information').

Mouse mixed cortical cell culture

To study excitotoxicity, primary cultures of mixed cortical cells containing both neurons and glia were prepared as described in Pellegrini-Giampietro *et al.* 1999 (details in 'Supporting information').

Immunoblotting

Total protein levels were quantified using the Pierce bicinchoninic acid Protein Assay, and the immunoblotting procedures were carried out as described in Bongers *et al.* (2007a). Briefly, cell cultures were harvested in lysis buffer and proteins (approximately 20 μg) separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were probed overnight with primary antibodies against one of the following antibodies: anti-Akt that specifically recognizes phosphorylation of Ser473 on Akt1, Akt2, and Akt3; anti-GSK-3β phosphorylated at Ser9, anti-CREB phosphorylated at Ser133; anti-Bcl-2, all used at 1 : 2000. Antibodies against phosphorylated proteins were dissolved in Tris-buffered saline (TBS) containing 1% bovine serum albumin, whereas antibodies against non-phosphorylated proteins were dissolved in TBS with 5% skimmed milk. Membranes were then washed with TBS and incubated for 90 min at 21°C in TBS/5% milk containing anti-rabbit peroxidase-conjugated secondary antibody (1 : 5000). After washing in TBS, enhanced chemiluminescence reaction (Pierce) was used to visualize the peroxidase-coated bands. Membranes were incubated in antibodies against β-actin (1 : 10 000 in TBS/5% milk) for 1 h at 21°C. Membranes were then washed with TBS and incubated for 90 min 21°C in TBS/5% milk containing anti-mouse peroxidase-conjugated secondary antibody (1 : 5000) and developed with enhanced chemiluminescence. Immunoreactive bands were detected by Immobilon Western Chemiluminescence horseradish peroxidase Substrate (Millipore) and quantified by densitometry analysis using an ImageQuant 350 imager and ImageQuant TL-1 software (GE Healthcare, Buckinghamshire, UK). All western blot target bands are expressed quantitatively by normalization to the control band on the same lane.

Immunohistochemistry

Cells cultures were grown on poly-L-lysine-coated glass coverslips and fixed in cold 4% paraformaldehyde for 4 h at 4°C, rinsed in TBS with 0.2% Triton X-100 (TBS-TX) and incubated in TBS containing 0.2%Tween 20 and 2% normal donkey serum (NDS) for 1 h at 21°C. Cells were then incubated in rabbit H_3R antibodies (1 : 500 in TBS-TX with 1% NDS) overnight at 4°C. After thorough rinsing in TBS-TX, cells were incubated in Cy3-conjugated donkey anti-rabbit IgG (1 : 800) in TBS containing 1% NDS for 90 min at 21°C. Rat cortical neurons were observed with an epifluorescence microscope (Olympus BX40; Olympus,

Hamburg, Germany). After rinsing, coverslips were mounted in antifading mounting medium. Mixed mouse cell cultures were incubated in H_3R antibodies as described above and then incubated in rabbit anti-GFAP antibodies diluted 1 : 1000 in phosphate-buffered saline with 0.2% Triton X-100 (PBS-TX) and 1% NDS overnight at 4°C. After rinsing in PBS-TX, cells were incubated in Alexa Fluor 488-conjugated anti rabbit (1 : 500 in PBS-TX, 1% NDS) for 90 min at 21°C. After rinsing, coverslips were mounted in antifading mounting medium. Confocal analysis was performed with a Leica TCS SP5 confocal scanning microscope (Leica, Mannheim, Germany), equipped with a HeNe/Ar laser and a Leica Plan Apo X63 1.4 oil immersion objective. To avoid bleed-through, green (Alexa 488) and red (Cy3) fluorescent signals were acquired sequentially, using the 488 nm and the 543 nm excitation wavelength, respectively. Series of optical sections (1024 × 1024 pixels, pixel size 204 nm) were collected through the cells at intervals of 400 nm. Confocal stacks were then filtered with a fast Fourier transform based bandpass filter to cut background noise (structures down to 20 μm and up to 204 nm) and then z-projected for visualization. Observations were performed on two sets of cell cultures from two different experiments. Each set of cell cultures consisted of 4–6 wells. Antibodies for the H_3R (kind gift of Drs Chazot and Shenton, Durham University, UK) are directed against residues 349–358 of the rat and human H_3R which are identical in the mouse sequence (Chen *et al.* 2003). The specificity of H_3R antibodies was previously described in Chazot *et al.* (2001) and Cannon *et al.* (2007). In the present study, elimination of H_3R primary antibodies resulted in no immunostaining (not shown).

Evaluation of rat cortical neuron culture viability

Primary cortical neurons [6–7 days *in vitro* (DIV)] were deprived of trophic factors (B27) for 24 h. Experimental groups were incubated for the whole period in the H_3R agonist immepip at the designated concentrations. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Cultures were incubated in MTT (0.5 mg/mL in PBS) for 30 min at 37°C. The supernatant was then removed and 200 μL dimethylsulfoxyde were added to each well. MTT metabolism was quantified spectrophotometrically at 490 nm in a microplate reader (Bio-Rad laboratories). Results were expressed as percentage MTT reduction, assuming that the absorbance of control cells was 100%.

Evaluation of mouse mixed cortical cells viability

At 14–15 DIV cultures were exposed to 300 μM NMDA for 10 min at 37°C. During NMDA exposure, the original culture medium was replaced with a HEPES controlled salt solution (composition in mM: 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 20 HEPES, 15 glucose, 10 NaOH and 10 mg/L phenol red). After the exposure to NMDA, cultures were rinsed with culture medium without serum, then the original medium was restored and cells were kept at 37°C, 100% humidity and 95% air/5% CO_2 atmosphere until neurodegeneration was assessed. The H_3R agonist immepip at the designated concentrations was added to the medium 1 h before NMDA, while the NMDA receptor antagonist MK-801 (10 μM) 15 min before NMDA and kept in the medium for the following 24 h. The H_3R antagonist/inverse agonist thioperamide (10 μM) was added to the medium 15 min prior to immepip and then co-incubated with immepip, whereas the phosphoinositide-3-kinase (PI3K) inhibitor LY 294002 (10 μM) was added to the medium 15 min prior to immepip and then co-incubated with it according to the protocol by Scartabelli *et al.* (2008). Cell damage was quantitatively evaluated by measuring the amount of lactate dehydrogenase (LDH) released from injured cells into the extracellular fluid 24 h following exposure to NMDA, as previously described (Pellegrini-Giampietro *et al.* 1999). Basal LDH release was determined in control cultures not exposed to NMDA and was subtracted from experimental values.

Measurement of caspase 3-like protease activity

The activity of caspase 3 in cortical cell cultures was measured as described in Meli *et al.* (2004) (details in 'Supporting information').

Statistical analysis

Statistical analyses were performed using JMP Statistical Discovery Software for MacIntosh (The Statistical Discovery Software, Cary, NC, USA). Differences among mean were evaluated by ANOVA, followed by Tukey–Kramer for all pairs *post hoc* test, unless otherwise stated. For all analyses, the null hypothesis was rejected at the 0.05 level.

Results

Expression of H_3R mRNA and protein in rat primary cortical neurons

Stable expression of H_3R mRNA in primary cortical neurons was demonstrated using a reverse-transcriptase polymerase chain reaction (RT-PCR; see Methods in Appendix S1) on total RNA of 4, 7 and 11 DIV neurons (Fig. S1a). In all subsequent experiments 7 DIV neurons were used. Expression of H_3R proteins was observed in 7 DIV rat cortical neurons using H_3R polyclonal antibodies (Fig. S1b). To identify the H_3R splice variants expressed in cortical neurons, specific primers were designed (see Methods in Appendix S1). Products identified as the functional splice variants $H_{3A–C}$ (Drutel *et al.* 2001) and the dominant negative splice variants $H_{3E,F}$ (Bakker *et al.* 2006) were detected in both 17 ED and 7 DIV rat cortical neurons. All five splice variants were expressed in the adult rat cortex as well (Fig. S1c and d).

Activation of the H_3R modulates Akt and GSK-3β phosphorylation

We previously showed that exposure of rat cortical neurons to nanomolar concentrations of the H_3R agonists immepip or RAMH resulted in a sustained and time-dependent increase in Akt phosphorylation at Ser473, an event known to be necessary for full activation of the kinase. H_3R-induced Akt activation reached plateau at 60 min (Bongers *et al.* 2007a). To study the intracellular events associated with Akt phosphorylation in 7 DIV cortical neurons, we measured immepip-induced Akt activation at this time point. First, we confirmed that 60 min treatment with 10 nM immepip

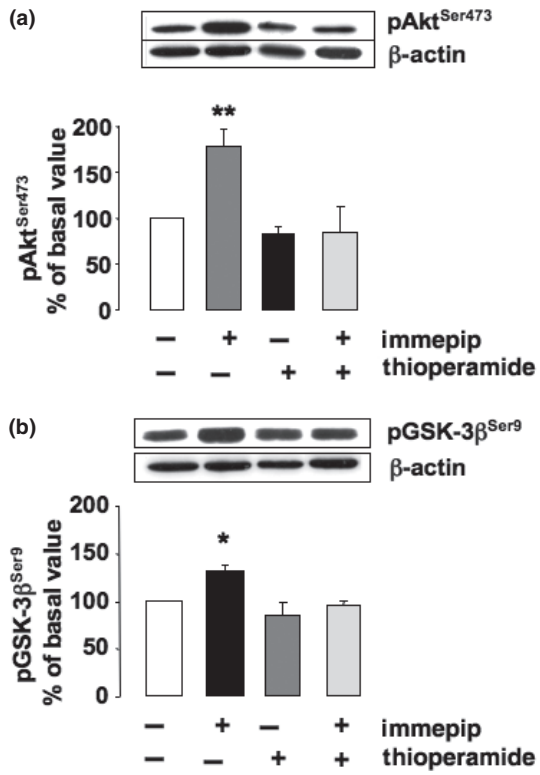

Fig. 1 Activation of the H_3R phosphorylates the Akt-GSK-3β signal transduction pathway. Seven DIV cortical neurons were incubated for 60 min in 10 nM immepip in the absence or presence of the H_3R inverse agonist/antagonist thioperamide (10 μM). Thioperamide was added to the incubation medium 10 min before immepip. (a) Representative western blots labeled with antibodies against Akt phosphorylated at Ser473 and quantification of the results obtained in five independent experiments. (b) Representative western blots labeled with antibodies against GSK-3β phosphorylated at Ser9 and quantification of the results obtained in four independent experiments. β-actin was used as loading control. $^{**}p < 0.001$; $^{*}p < 0.05$ versus all other groups; ANOVA with Tukey–Kramer *post hoc* test for multiple comparisons.

increased Akt phosphorylation at Ser473 (178 ± 17% of basal; $n = 5$, $p < 0.01$), as analyzed by specific anti-phospho-Akt immunoblots (Fig. 1a). Immepip-mediated phosphorylation of Akt was inhibited by co-incubation with the H_3R antagonist/inverse agonist thioperamide (10 μM; 85 ± 11% of basal). Thioperamide produced a small but not significant reduction of basal Akt phosphorylation when administered alone (83 ± 14% of basal), indicating absence of constitutive activity of the H_3R at this intracellular signaling pathway in cultured rat cortical neurons. We previously reported that in SK-N-MC neuroblastoma cells expressing the human H_3R, stimulation with immepip resulted in phosphorylation of GSK-3β at Ser9 (Bongers *et al.* 2007a). In 7 DIV cortical neurons as well, stimulation with 10 nM immepip for 60 min increased significantly phosphorylation of GSK-3β at Ser9, as revealed by anti-phospho-GSK-3β immunoblots (132 ± 5% of basal; $n = 4$; $p < 0.05$; Fig. 1b). The time course of GSK-3β inactivation paralleled that of Akt phosphorylation (not shown). Thioperamide (10 μM) completely inhibited immepip-induced inactivation of GSK-3β (95 ± 6% of basal). When administered alone, 10 μM thioperamide did not change basal phosphorylation of GSK-3β Ser9 significantly (86 ± 13% of basal).

Phosphorylation of the Akt/GSK-3β axis depends on PI3K and MAP kinase activation

To understand the mechanism by which H_3R activation mediates Akt phosphorylation, cortical neurons were treated with LY 294002 at concentrations commonly used to block PI3 kinase (Davies *et al.* 2000) and to inhibit H_3R-mediated phosphorylation of Akt at Ser473 in SK-N-MC transfected cells (Bongers *et al.* 2007a). As shown in Fig. 2(a), immepip increased Akt phosphorylation significantly (168 ± 15% of basal; $n = 3$; $p < 0.01$), 30 min pre-incubation with 20 μM or 50 μM LY 294002 decreased significantly basal Akt phosphorylation at Ser473 and prevented immepip-induced increase of Akt activation. Pre-incubation of cortical neurons for 30 min with LY 294002 blocked both basal and immepip induced GSK-3βSer9 phosphorylation (178 ± 36% of basal; $n = 3$) in a manner comparable to LY 294002 effect on Akt activation (Fig. 2a). Treatment with a specific mitogen-activated protein kinase kinase (MEK) inhibitor also blocked immepip-mediated increase of Akt activation. A 30 min pre-treatment with U0126 (10 μM) had no effect on basal Akt phosphorylation, but completely abolished immepip-induced Akt phosphorylation (281 ± 47%; $n = 3$; $p < 0.01$; Fig. 2b).

Activation of the H_3R has no effect on CREB phosphorylation, but modulates Bcl-2 expression.

Downstream targets of the PI3K/Akt pathway are transcription factors such as CREB that is believed to play a key role in promoting neuronal survival (Lonze and Ginty 2002). Incubation of 7 DIV cortical neurons with 10 nM immepip up to 60 min did not modify significantly CREB phosphorylation at Ser133 at any time point analyzed, as shown using specific anti-phospho-CREB immunoblots (Fig. S2; $n = 3$). In view of a potential effect of H_3R activation on Bcl-2 expression, rat cortical neurons were treated with 10 nM immepip for increasing periods of time. A significant increase of Bcl-2 expression (139 ± 5% of basal; $n = 4$; $p < 0.05$) was observed after 2 h incubation with immepip (Fig. 3a). Bcl-2 expression declined to baseline levels within 24 h. The increase of Bcl-2 expression induced by 2 h immepip stimulation (142 ± 14%; $p < 0.05$) was blocked by co-incubation with 10 μM thioperamide (107 ± 10%; $n = 4$) that *per se* had no effect (100 ± 16%; Fig. 3b, left panel). To understand whether immepip-induced increase of Bcl-2 expression depends on

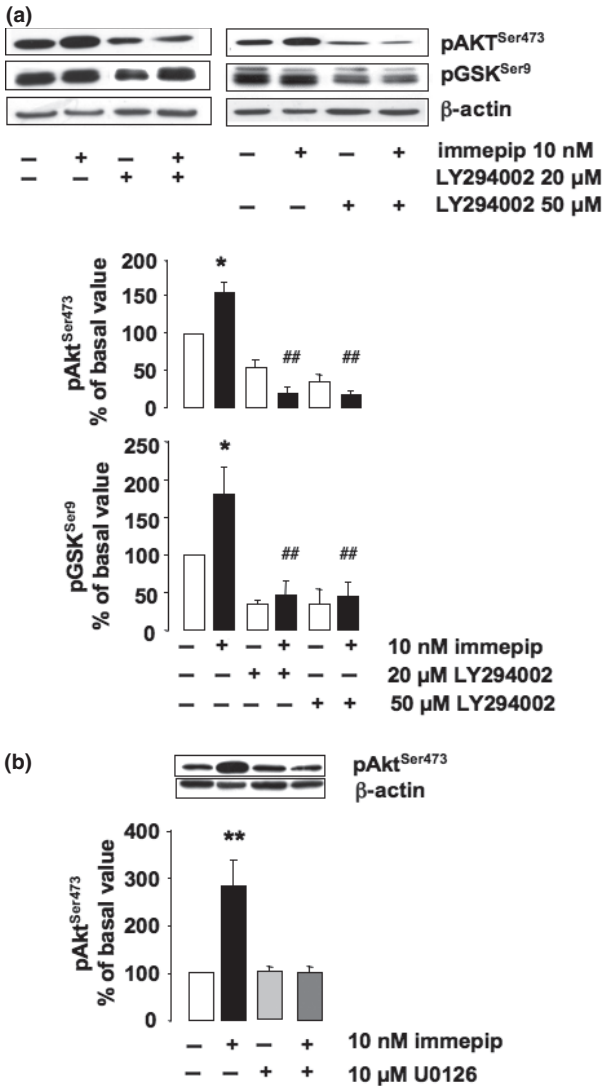

Fig. 2 Effect of PI3K and MEK inhibitors on H_3R-induced activation of Akt/GSK3β. (a) Seven DIV rat cortical neurons were incubated for 60 min in 10 nM immepip in the absence or presence of the PI3K inhibitor LY 294002, pre-incubated for 30 min at the indicated concentrations. Representative western blots labeled with antibodies against Akt phosphorylated at Ser473 and GSK3β phosphorylated at Ser9, and quantification of the results obtained in three independent experiments. (b) Seven DIV rat cortical neurons were incubated for 60 min in 10 nM immepip in the absence or presence of the MEK inhibitor U0126 (10 nM), pre-incubated for 30 min. Representative western blots labeled with antibodies against Akt phosphorylated at Ser473 and quantification of the results obtained in three independent experiments. β-actin was used as loading control. $^{**}p < 0.01$; $^{*}p < 0.05$ relative to control; $^{\#\#}p < 0.01$ relative to immepip; ANOVA with Tukey–Kramer *post hoc* test for multiple comparisons.

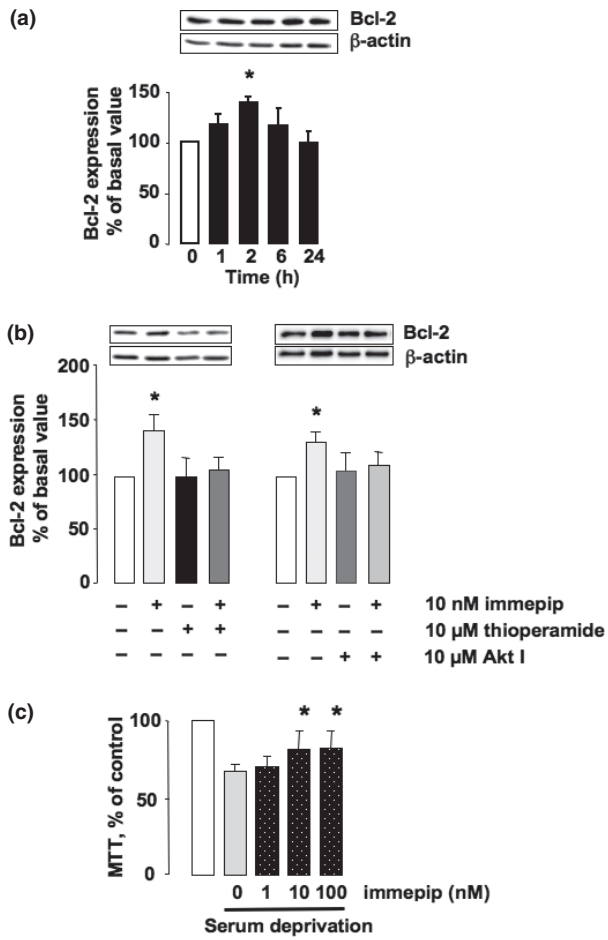

Fig. 3 Activation of the H_3R increases the expression of Bcl-2 and protects neurons from serum deprivation-induced injury. (a) Seven DIV cortical neurons were incubated with 10 nM immepip for the indicated periods of time. Representative western blots labeled with antibodies against Bcl-2 and quantification of the results obtained in four independent experiments. $^{*}p < 0.05$ relative to control; ANOVA with Dunnett's *post hoc* test. (b) Seven DIV cortical neurons were incubated for 2 h in 10 nM immepip in the absence or presence of the H_3R inverse agonist/antagonist thioperamide (10 μM; left panel), or an Akt inhibitor (Akt I, 10 mM; right panel). Thioperamide was added to the incubation medium 10 min before immepip, whereas Akt I was added to the incubation medium 4 h before immepip. $^{*}p < 0.05$ versus all groups; ANOVA with Tukey–Kramer *post hoc* test for multiple comparisons. (c) Seven DIV neurons were deprived of serum in the medium for 24 h during which they were incubated with immepip at the designated concentrations. Neural injury was assessed by the MTT assay and expressed as percentage of neuronal injury relative to untreated cells. $^{*}p < 0.05$ relative to non-treated, serum deprived neurons; ANOVA with Tukey–Kramer *post hoc* test for multiple comparisons.

Akt activation, cortical neurons were pre-incubated for 4 h with 10 μM of the Akt inhibitor, 1L-6-hydroxymethyl-chiroinositol 2-(*R*)-2-O-methyl-3-O-octadecylcarbonate (Akt I; Hu *et al.* 2000). The Akt I diminished immepip-induced increase of Bcl-2 expression (106 ± 15% vs. 132 ± 8% of basal; $n = 3$; $p < 0.05$; Fig. 3b, right panel). The Akt I did not modify Bcl-2 basal expression significantly when administered alone (110 ± 12%).

Neuroprotective effect of immepip against serum deprivation-induced cell death of cortical neurons

To test the physiological significance of H_3R-induced activation of the Akt/GSK-3β axis, 7 DIV cortical neurons were maintained in serum-free medium for 24 h, a procedure that induces significant cell death, in the presence of increasing concentrations of immepip. In four out of nine cases, serum deprivation caused approximately 30% cell death, as evidenced by MTT reduction, a viability assay that measures activity of mitochondrial succinate dehydrogenase (cell viability was 68 ± 4% of controls with serum). When neurons were exposed to either 10 or 100 nM immepip, cell injury was significantly different from serum-deprived, non-treated neurons ($p < 0.05$; Fig. 3c). Incubation with 1 nM immepip was without effect. In the remaining five cases, 24 h serum deprivation caused extensive cell damage (over 50% of controls with serum) and immepip treatment was ineffective (not shown). Hence, given the difficulty to carry out the pharmacological characterization of H_3R-induced neuroprotective effects in primary cultures of rat cortical neurons, we tested cell survival in another model of neurotoxic damage, NMDA-induced excitotoxicity in mixed cultures of mouse cortical cells. In fact, considerable experience in our laboratory established that NMDA-induced excitotoxicity is more reliably accomplished and quantitatively assessed in cultured mouse mixed cortical cells rather than in pure rat cortical neuron cultures (Pellegrini-Giampietro *et al.* 1999; Meli *et al.* 2004).

Neuroprotective effects of H_3R agonists against NMDA-induced neurotoxicity in mixed mouse cortical cells

We first determined whether cultured mouse cortical neurons express H_3R proteins and whether immepip induces Akt phosphorylation in this preparation. Cortical neurons were grown for 14 days on mouse glial cells before proceeding to double immunostaining with GFAP and H_3R antibodies. Figure 4(a1) shows GFAP immuno-positive glial cells and a dense H_3R immunopositive signal on somata and dendrites of cortical neurons (Fig. 4a2). Dense H_3R immunopositive product was also observed within the cytoplasm of cortical neurons. The merged photomicrograph of Fig. 4(a3) shows that H_3R immunostaining is localized on cortical neurons. One hour incubation of mouse cortical cells in 100 nM immepip increased Akt phosphorylation at Ser473 (150 ± 8% of baseline values; $n = 5$; $p < 0.05$; Fig. 4b) and the effect was inhibited by adding 10 μM thioperamide to the incubation medium (111 ± 12%). Thioperamide *per se* had no significant effect (117 ± 11%), indicating absence of constitutive activity of the H_3R at this signaling pathway, as observed in rat cortical neurons. Exposure of mouse cortical

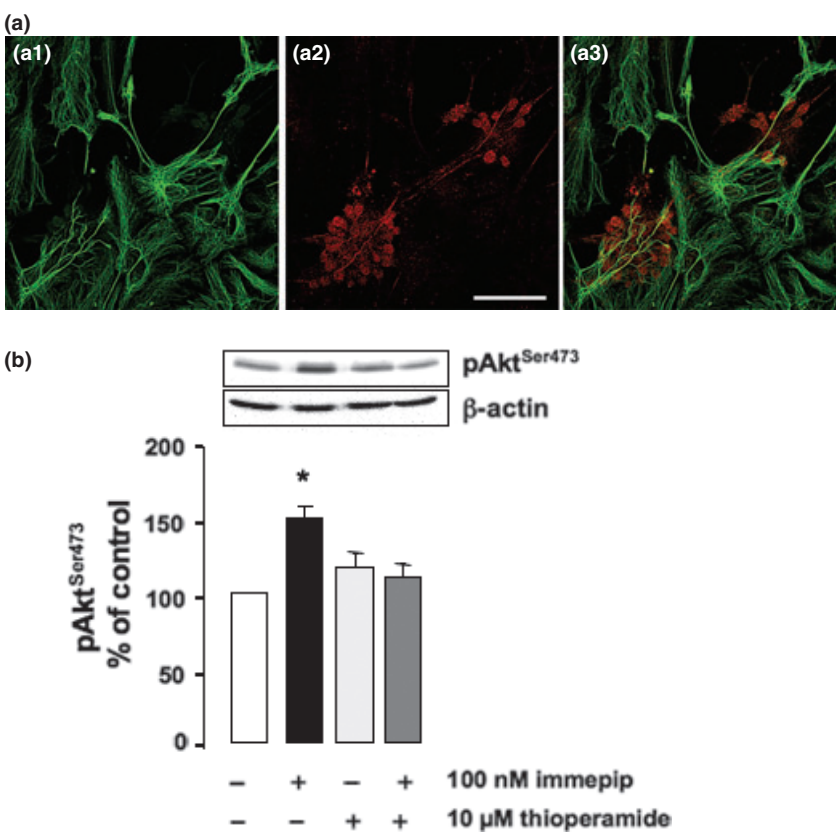

Fig. 4 H_3R are expressed in mouse cortical cells in culture and their activation increases Akt phosphorylation. (a) Photomicrographs showing the distribution of anti-H_3R immunopositive product in mixed cultures of mouse embryonic glial and neural cells. Mixed cultures were immunolabeled with both anti-GFAP and H_3R antibodies; (a1) Glial cells show intense anti-GFAP immunopositive product. (a2) Immunoreactivity for the H3R in the same preparation as in a1; (a3) merged double labeled images. Scale bar = 50 μm. (b) Mixed mouse cortical cells were incubated for 1 h in 10 nM immepip in the absence or presence of the H_3R inverse agonist/antagonist thioperamide (10 μM). Thioperamide was added to the incubation medium 10 min before immepip. Top: representative western blots labeled with antibodies against Akt phosphorylated at Ser473. Bottom: quantification of the results obtained in five independent experiments. *$p < 0.05$ versus all other groups; ANOVA with Tukey–Kramer *post hoc* test for multiple comparisons.

cells to 300 μM NMDA for 10 min caused neural injury associated with substantial efflux of LDH in the bathing medium (approximately 4.5-fold from the basal level). Using LDH release as a quantitative index, we measured the extent of neuronal death in the presence of immepip. NMDA-induced LDH release was reduced in a dose dependent manner when immepip was added for 24 h to the medium ($n = 4$; Fig. 5a). Also, another H_3R agonist RAMH (100 nM) significantly reduced NMDA-induced LDH production. The neuroprotective effect of either 100 nM immepip or RAMH (74 ± 2% and 71 ± 1%, respectively, of value in the presence of NMDA; $p < 0.01$) was blocked by pre-incubation with 10 μM thioperamide (100 ± 13% and 93 ± 13%, respectively). Thioperamide *per se* had no significant effect (98 ± 4%). As previously reported (Pellegrini-Giampietro *et al.* 1999), the NMDA receptor antagonist MK-801 (10 μM) significantly reduced (56 ± 4%) NMDA-induced LDH release. The PI3K inhibitor LY 294002 had no effect on NMDA toxicity, but prevented the reduction in NMDA-induced neurotoxicity observed after 24 h exposure of cortical cells to 100 nM immepip (58 ± 7%; $p < 0.01$; Fig. 5b). The activation of caspase 3 has a key role in the initiation and the execution of NMDA-induced apoptosis (Tenneti and Lipton 2000). In cortical mixed cell cultures, we measured the activity of caspase 3 following exposure to NMDA**.** Addition to the medium of 300 μM NMDA for 10 min increased caspase 3 activity approximately two times the basal levels. When immepip (100 nM) was added to the medium 1 h before exposing cell cultures to NMDA and maintained for the following 6 h, caspase 3 activity was reduced to 22.5 ± 0.3% of the value in the presence of NMDA ($p < 0.01$; $n = 4$; Fig. 5c). Thioperamide (10 μM) blocked the effect of immepip, as caspase 3 activity was not significantly different from the value obtained in NMDA alone. Furthermore, thioperamide did not significantly modify NMDA-induced increase of caspase 3 activity.

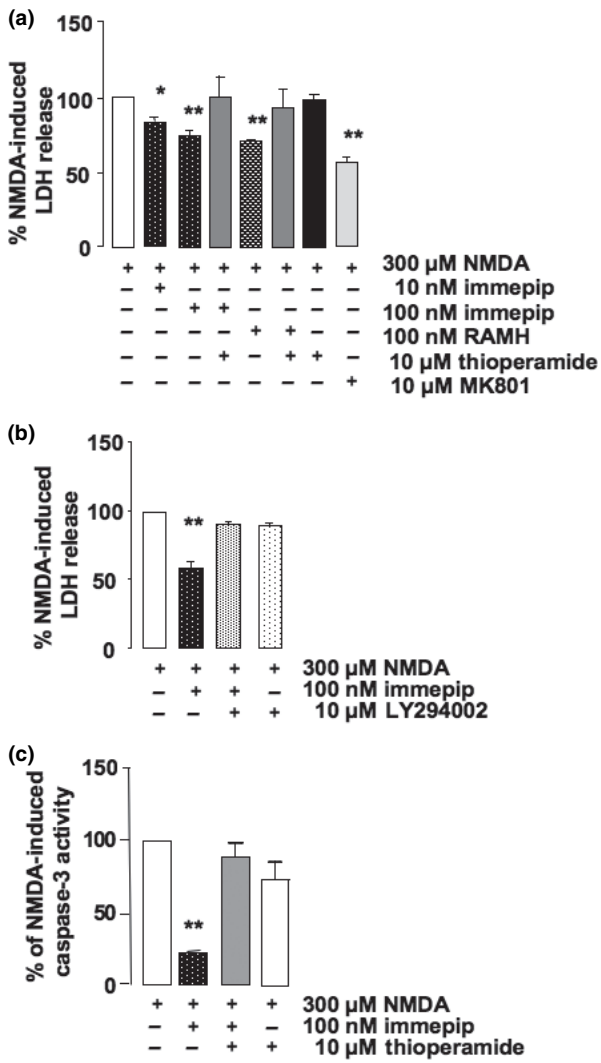

Fig. 5 Activation of the H_3R reduces neuronal death following NMDA exposure in mixed mouse cortical cultures and modulates caspase activity. (a) Immepip or R-α-methyl histidine (RAMH) were added to the culture 1 h before a 10 min NMDA exposure and maintained in the medium for the following 24 h in the absence or presence of the H_3R inverse agonist/antagonist thioperamide. The NMDA receptor antagonist MK801 was added 1 h before NMDA exposure and maintained in the medium for the following 24 h. (b) Immepip was added to the culture 1 h before a 10 min NMDA exposure and maintained in the medium for the following 24 h in the absence or presence the PI3K inhibitor LY 294002. Cell death was assessed by measuring the release of LDH in the medium 24 h after NMDA exposure. Data are expressed as percentage of control LDH release in the presence of NMDA and represent the mean ± SEM of four/five independent experiments in A and three independent experiments in (b). (c) Caspase 3 activity was measured in the supernatant of lysed cells after 6 h incubation in H_3R ligands. Data are expressed as percentage of caspase 3 basal activity in NMDA-treated cells and represent the mean of ±SEM of four experiments. ANOVA with Tukey–Kramer *post hoc* test for multiple comparisons. $^{**}p < 0.01$; $^{*}p < 0.05$ relative to NMDA-treated cells.

Discussion

We recently demonstrated that the H_3R-mediated activation of Akt occurs in primary cultures of rat cortical neurons (Bongers *et al.* 2007a), but the physiological relevance of such finding was not explored. In this study, we show that H_3R activation induces phosphorylation of the Akt/GSK-3β pathway, increases the expression of the antiapoptotic protein Bcl-2, and protects neurons against neurotoxic insults. In cultured rat and mouse cortical cells, Akt activation was visualized by increases in phosphorylation of Akt at Ser473 using site-specific antibodies for the phosphorylated kinase. In rat cortical neurons H_3R activation led to phosphorylation of Ser9, hence inactivation, of the Akt downstream target GSK-3β. The H_3R-mediated phosphorylation of Akt depended on PI3K and, unexpectedly, on MEK1/2 activation, as incubation of cortical neurons with specific inhibitors

of either kinase completely prevented immepip-induced Akt phosphorylation. These results contrast with previous reports indicating that U0126 did not block H_3R-mediated increase of Akt phosphorylation in SK-N-MC cells transfected with the human H_3R (Bongers *et al.* 2007a). Incubation of cortical neurons with the PI3K inhibitor LY 294002 blocked not only immepip-induced phosphorylation of Akt/GSK-3β, but inhibited the basal levels of active Akt as well. Presumably LY 294002 binds to and inhibits the catalytic activity of active PI3K, with the results that basal phosphorylation of Akt occurring during the experiment will be blocked. This is suggestive of a high degree of basal activation of the Akt/GSK-3β axis in cultured rat cortical neurons. Treatment of cortical neurons with the MEK inhibitor U0126 abolished immepip-evoked phosphorylation of Akt, whilst having no effect on basal Akt activity. Thus, the MEK pathway appears to be involved in the H_3R-mediated activation of the PI3K/Akt axis suggesting the existence of a cross-talk between these two signaling pathways in cultured rat cortical neurons. Cross-talk between extracellular signal-regulated kinase 1/2 (ERK1/2, the downstream targets of MEK) and PI3K/Akt pathways has been reported also following NMDA receptor stimulation in rat striatal neurons (Perkinton *et al.* 2002). We did not investigate the effect of H_3R stimulation on the phosphorylation of ERK1/2. H_3R-dependent activation of this signal transduction pathway was demonstrated in COS-7 cells expressing the rat H_3R (Drutel *et al.* 2001), but not in rat hippocampal slices, where H_3R agonists failed to phosphorylate ERK1/2 directly (Giovannini *et al.* 2003). Whether PI3K and ERK1/2 cascades have common downstream targets and synergistic effects in, e.g. neuroprotective or plasticity mechanisms, are questions worth being addressed.

Constitutive H_3R activation of Akt was observed in SK-N-MC transfected with the human H_3R (Bongers *et al.* 2007a). This was not observed in primary cultures of rat cortical neurons, nor in mouse cortical cell cultures, as the H_3R antagonist/inverse agonist thioperamide had no effect on basal Akt/GSK-3β phosphorylation, nor on Bcl-2 expression. As discussed previously in Bongers *et al.* (2007a), the discrepancy may be explained in terms of differences between the human and rat H_3R that shows lower level of constitutive activity (Wieland *et al.* 2001; Bongers *et al.* 2007b), or in terms of low expression of functional H_3R isoforms ($H_{3a,b,c}$) in cortical neurons, not sufficient to observe constitutive activity at the Akt pathway. In this regard we found that cultured rat cortical neurons expressed the same complement of functional H_3R splice variants ($H_{3A,B,C}$) as the parent 17 ED and adult cortical neurons. Hence, the neuroprotective effect of immepip is most likely not isoform-specific.

H_4R are present in the rat cortex (Chazot *et al.* 2007; Strakhova *et al.* 2009). Immepip binds to both H_3R and H_4R with pEC_{50} values of 10.4 ± 0.1 and 7.8 ± 0.1, respectively (Lim *et al.* 2005). Although the possibility exists that the observed responses in our cell system are partially mediated via H_4R, the concentration of immepip used in the present study (10 nM) presumably was insufficient to fully activate H_4R. Further investigation, although, is required to establish H_4R signaling properties and its contribution to neuroprotection.

The Akt pathway has been implicated in regulating several important cellular processes, including cell plasticity and survival, proliferation and metabolism. Akt promotes neuronal cell survival and opposes apoptosis by a variety of routes, e.g. modulating inhibitors of apoptosis such as Bcl-2 and Bcl-x (Song *et al.* 2004). PI3K/Akt-dependent Bcl-2 up-regulation was demonstrated in mesenchymal stem cells treated with brain derived neurotrophic factor (BDNF) that induces neural differentiation and cell survival (Lim *et al.* 2008). A similar mechanism was proposed for the neuroprotective effect of estradiol on striatal dopaminergic neurons in a mouse model of Parkinson's disease (D'Astous *et al.* 2006). In rat cortical neurons, activation of the H_3R with nanomolar concentrations of immepip increased the expression of the antiapoptotic protein Bcl-2, an effect that depended on Akt activation. In rat cortical neurons, immepip did not significantly affected CREB phosphorylation at Ser133. CREB activation, although, is crucially dependent on increased intracellular cAMP and phosphorylation at Ser133 by protein kinase A (Gonzalez and Montminy 1989). H_3R activation couples negatively to adenylyl cyclase in a variety of heterologously transfected cell lines and in rat striatal slices (reviewed in Bongers *et al.* 2007c). Hence, H_3R activation may reduce or increase CREB phosphorylation via independent pathways that may cancel each other out. We then explored the possibility that H_3R mediated activation of antiapoptotic mechanisms had neuroprotective effects in cultured cortical neurons. H_3R agonists protected cortical cells from neurotoxic insults in the same range of concentrations that activated the Akt/GSK-3β axis and increased Bcl-2 expression. The role of the H_3R-PI3K-Akt signaling pathway in protecting mouse cortical cells from NMDA-induced neurotoxic insult was demonstrated by the experiments showing that neuroprotection afforded by immepip was completely abolished by an inhibitor of PI3K, and hence Akt, activity. In addition, immepip blocked NMDA-induced activation of caspase 3 suggesting that immepip exerts a neuroprotective effect by blocking apoptosis. This is the first report of the potential antiapoptotic characteristics of H_3R agonists.

There is evidence that activation of the H_3R may be implicated in neuroprotection as H_3R mRNA is up-regulated following induction of cerebral ischemia (Lozada *et al.* 2005) or kainic acid induced seizures in the rat (Lintunen *et al.* 2005), although in brain regions other than the neocortex. Our results contrast with a report by Dai *et al.* (2006) who showed that the H_3R antagonist thioperamide attenuates the necrosis of cortical neurons induced by

exposure to 100 μM NMDA for 3 h, by enhancing GABA release. Cell viability tests were carried out immediately after NMDA exposure, whereas in our protocol cells exposed to NMDA for 10 min were incubated in histaminergic compounds for 24 h before the viability studies. Previous studies showed that mild or intense NMDA exposures elicit apoptotic or necrotic cell death, respectively, hence different intracellular signaling pathways are activated (Pellegrini-Giampietro *et al.* 1999; Meli *et al.* 2004). Other methodological differences such as extraction of neurons from postnatal day 1 pups that were kept in culture for 12–14 days, may account for the discrepancy between the data by Dai *et al.* (2006) and our results.

Much of the recent interest in developing new ligands of the H_3R stems from the potential use of H_3R antagonists in controlling feeding behavior, disorders of the sleep-wake cycle and cognitive impairments associated with Alzheimer's or Parkinson's disease (reviewed in Sanders *et al.* 2008). However, there are potential therapeutic applications for H_3R agonists as well. H_3R activation in the CNS results in lower hypothalamic histamine release and H_3R agonist may be used against insomnia (Lin 2000). Also, Hough and co-workers have revealed an antinociceptive role for spinal histamine H_3R (Cannon and Hough 2005). Our observations that H_3R agonists activate the PI3K/Akt pathway and have a neuroprotective effect in cultured neurons may have relevance in the prevention of, for instance, ischemic neuronal damage or neurodegenerative diseases. Also, schizophrenic patients have impaired Akt/GSK-3β signaling (Emamian *et al.* 2004) and evidence points to a key role for GSK-3β in promoting neurodegeneration (Kaytor and Orr 2002). Indeed, GSK-3 is involved in a cascade of events, such as hyperphosphorylation of tau protein, increased production of β-amyloid, local cerebral inflammatory responses that may culminate in Alzheimer's disease (Hooper *et al.* 2008). In this regard, binding studies showed that the expression of H_3R is spared in the brain of Alzheimer's patients (Medhurst *et al.* 2007). To fully understand the impact of H_3R-induced activation of antiapoptotic pathways in the CNS, *in vivo* experiments are necessary, the more so as H_3R antagonists are now viewed as potential therapeutics for schizophrenia (Ligneau *et al.* 2007) and Azheimer's disease (Medhurst *et al.* 2007).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary Material and methods.

Figure S1. Primary cultures of embryonic rat cortical neurons express selective H_3R mRNA.

Figure S2. Activation of the H_3R does not modify CREB phosphorylation.

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